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14. ABSTRACT While current DC vaccines are safe, their antitumor activity is limited. This is foremost due to the presence of regulatory T cells (Tregs), which create an immunosuppressive environment in breast cancer patients. In addition, there is increasing evidence that effective solid tumor vaccines have to target cancer cells as well as their supporting stroma. Thus, overcoming Treg mediated immune suppression and targeting the tumor stroma in addition to breast cancer cells may produce the desired increase in antitumor activity of DC vaccines for breast cancer. According to the task 1 in the approved statement of work, recombinant lentiviral vector expressing A20-shRNA, HER2 and FAP was prepared and 4T1.2-neu tumor bearing mice were immunized with the lentivirus transduced DC vaccine. We found the DC vaccine induced robust T cell responses against HER2 and FAP, resulting in enhanced antitumor effect. Thus, the DC vaccine that target not only HER2, but also Treg and FAP, might present the optimized DC vaccine strategy against HER2+ breast cancer.					
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Introduction

The intent of this project is to develop an effective DC vaccine strategy for patients with breast cancer. While current DC vaccines are safe, their antitumor activity is limited (1-3). This is foremost due to the presence of regulatory T cells (Tregs), which create an immunosuppressive environment in breast cancer patients (4-11). In addition, there is increasing evidence that effective solid tumor vaccines have to target cancer cells as well as their supporting stroma (12-20). Therefore, we hypothesize that overcoming Treg mediated immune suppression and targeting the tumor stroma in addition to breast cancer cells may produce the desired increase in antitumor activity of DC vaccines for breast cancer.

In our previous study, we have demonstrated that silencing the zinc-finger A20 in DCs overcomes Treg-mediated immunosuppression in tumor bearing mice using ovalbumin as a model antigen (21). Since effective Treg depletion prior to vaccination has proven difficult, A20-silenced DC vaccine might represent an alternative strategy to overcome Treg-mediated immunosuppression. In this project we now propose to develop an A20-silenced DC vaccine to activate breast cancer- and stroma-specific cytotoxic T lymphocytes (CTL), while concomitantly inhibiting Tregs. We will target the human epidermal growth factor receptor (HER)2 expressed on breast cancer cells and the fibroblast activating protein (FAP) expressed on the supporting tumor stroma.

In the first year of this award, recombinant lentiviral vector expressing shA20, FAP, and HER2 (Lv-shA20-FAP-HER2) was prepared and immunized to 4T1.2-neu tumor bearing mice. First, Lv-shA20-FAP-HER2 and control vectors (Lv-shA20-FAP, Lv-shA20-HER2, Lv-shGFP-FAP, Lv-shGFP-HER2, Lv-shGFP-FAP-HER2) were prepared and transduced to mice bone-marrow derived DCs. Then these DC vaccines were immunized to Balb/c mice. The results indicated that immunization of A20-silenced DC vaccine induced more robust T cell responses against FAP or HER2, compared to that of GFP-silenced DC vaccine. In addition, the immunization of DC-shA20-FAP-HER2 inhibited 4T1.2-neu growth more efficiently, compared to that of control vaccines. Thus, DC-shA20-FAP-HER2 is an optimized DC vaccine strategy against breast cancer. To better investigate the effect of DC-shA20-FAP-HER2 against breast cancer, in the second year of this award we will evaluate these DC vaccines using HER2 transgenic mice.

Body

Task 1: To construct lentiviral vector coexpressing A20-shRNA, HER2, and FAP and evaluate the potency of DC-A20-shRNA-HER2-FAP to induce HER- and FAP-specific CTL and Th responses in 4T1.2-Neu bearing mice.

Preparation of animal protocol. Animal protocol AN3288 of “Immunization by DC vaccine” was amended to include the immunization of DC-shA20-FAP-HER2 and other control DC vaccines for the study and approved by Baylor College of Medicine.

Generation of recombinant lentiviral vector expressing A20-shRNA, HER2 and FAP. The recombinant lentiviral vectors expressing A20-shRNA, HER2 and FAP, including Lv-shA20-HER2-FAP, Lv-shGFP-HER2-FAP, Lv-shA20-HER2, Lv-shA20-FAP, Lv-shGFP-HER2, Lv-shGFP-FAP, and Lv-shA20 were prepared. We generated an expression cassette containing A20 shRNA, HER2 and FAP by PCR cloning (Fig.1). The lentiviral vector used in this study was pTRIPΔU3 CMV eGFP (21-24), which is self-inactivating (SIN vectors) with a 400 bp deletion in the U3 region of the 3' long terminal repeat (LTR), and contains a 178-bp fragment encompassing the central polypurine tract (cPPT) and the central termination sequence (CTS). pTRIP-H1-BY-W vector was generated from pTRIPΔU3CMV eGFP for expression of siRNA from the H1 RNA promoter and co-expression of a bicistronic blasticidin resistance/eYFP selection marker. Murine FAP gene was kindly provided by Dr. J. Cheng (FCCC, PA). Recombinant Lentiviral vectors were generated by standard methods established in our laboratory. All vectors were verified by DNA sequencing. Recombinant pseudotyped lentiviral vectors were generated by co-transfection of three plasmids into 293 cells and concentrated by ultracentrifugation, as described previously.

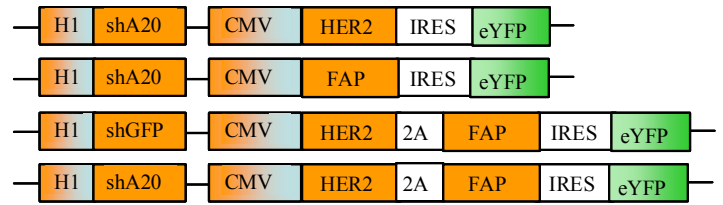


Fig. 1: Scheme of Expression cassettes encoding shA20, FAP, and HER2

Characterization of genetically modified DC functions *in vitro*. We transduced bone-marrow derived DCs from Balb/c mice with Lenti-shA20-HER2-FAP, or controls. Mouse bone marrow (BM)-derived DCs were prepared as described in our previous study (21-24). Briefly, mouse bone marrow was flushed from limbs, passed through a nylon mesh, and depleted of red cells with ammonium chloride. After extensive washing with RPMI-1640, cells were cultured with 2.5 ml of RPMI-1640 supplemented with 10% FBS, mGM-CSF/ml (20 ng/ml) and recombinant mouse IL-4 (20 ng/ml; PeproTech). On days 2 and 4 of culture, the supernatant was removed and replaced with fresh media containing 20 ng/ml of rmGM-CSF and 20 ng/ml of rmIL-4. All cultures were incubated at 37°C in 5% humidified CO₂. Nonadherent granulocytes were removed after 48 hr of culture and fresh medium was added. After 7 days of culture, >80% of the cells expressed characteristic DC-specific markers as determined by FACS. Transduction of BM-derived DCs with these LV vectors routinely yielded 30-60% of cells (data not shown). A20 expression in Lv-shA20 transduced DC was downregulated by 80-90% in comparison to DCs transduced with the control vector (data not shown). FAP and HER2 expression of transduced BM-DCs was confirmed by RT-PCR.

Evaluation of the potency of the resultant vaccine to induce HER2- and FAP-specific CTL responses in 4T1.2-neu bearing mice models. *shA20-DC vaccines induce potent FAP- or HER2-specific immune response.* We tested whether A20-silenced DC vaccine strategy was able to induce enhanced FAP- and HER2-specific T cell responses. Balb/C mice were immunized with DC vaccines or PBS, and the functional status of CD8⁺ and CD4⁺ T cells was then evaluated using interferon (IFN)- γ ELISPOT assays. We observed that mice immunized with A20-silenced DC vaccines had significantly higher frequencies of IFN- γ ⁺ CD8⁺ T (Fig. 2) and CD4⁺ T cells (Not shown), compared to those given DC-shGFP vaccines. These data demonstrate a critical role for A20 in control of the magnitude of FAP- and HER2-specific T-cell responses.

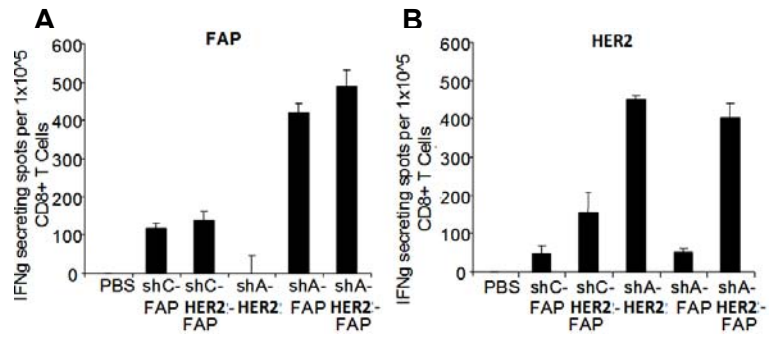


Fig. 2. ELISPOT assays of T-cell responses against FAP and HER2. Mice were immunized with DC vaccines or PBS. Isolated CD8⁺ T cells from immunized mice (2-3/group) were subjected to IFN γ ELISPOT assays against FAP (A) and HER2 (B)

DC-shA20-FAP-HER2 vaccination inhibits 4T1-neu progression. We investigated whether DC-shA20-FAP-HER2 may induce more potent anti-stroma and anti-tumor immunity with the ability to control the growth of tumors. DC vaccines were immunized to the Balb.C mice followed by inoculation of 4T1.2-neu tumor in the fourth mammary fat pad in 7 days later (Fig. 3). By targeting both FAP and HER2, one immunization of DC-shA20-FAP-HER2 completely prevent the progression of 4T1.2-neu tumor in 80 days. These results demonstrate the DC-shA20-FAP-HER2 has potent antitumor activity.

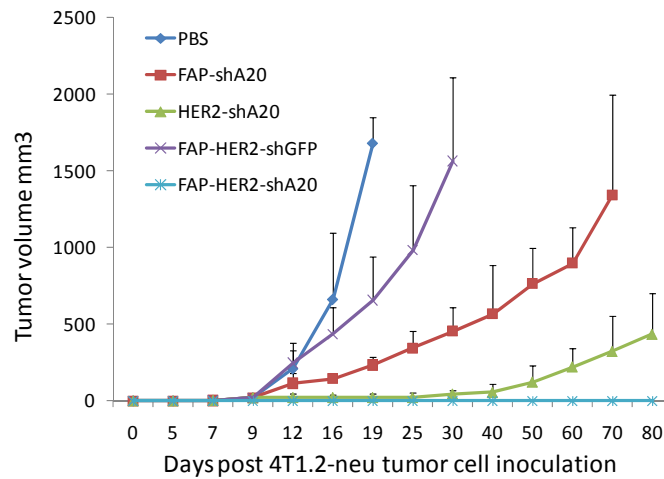


Fig.3: Comparison of antitumor activity. Mice were immunized with 1x10⁶ DCsor PBS control followed by inoculation of 4T1.2-neu cells (5x10⁵) 7 d later .

DC-shA20-FAP vaccine decreases immunosuppressive cells in the tumor. We investigated whether DC-shA20-FAP vaccination results in a decrease in suppressive cells in the tumors. While we are evaluating our vaccine using 4T1.2-neu mice model, we also used B16 mice model. Staining B16 tumors for CD11b identified a large population of infiltrating macrophages (Fig.4A), which significantly decreased after vaccination of FAP-targeted DC vaccine. We observed that both the CD11b⁺Gr1^{dim} population and CD11b⁺Gr1^{hi} population were significantly decreased after vaccination of FAP-targeted DC vaccine from 41.38% to 26.44% and 4.37% to 2.48%, respectively. In addition, upregulation of CD80 expression on tumor infiltrating CD11c⁺ population was observed in the recipients of FAP-targeted DC vaccine (Fig.4B). Taken together, these data indicate that our FAP-targeted DC vaccine decreases immature DC and tumor-associated macrophages, supporting our hypothesis in Aim 1 that the A20-silenced FAP-targeted DC vaccine has the potential to reverse the suppressive tumor microenvironment.

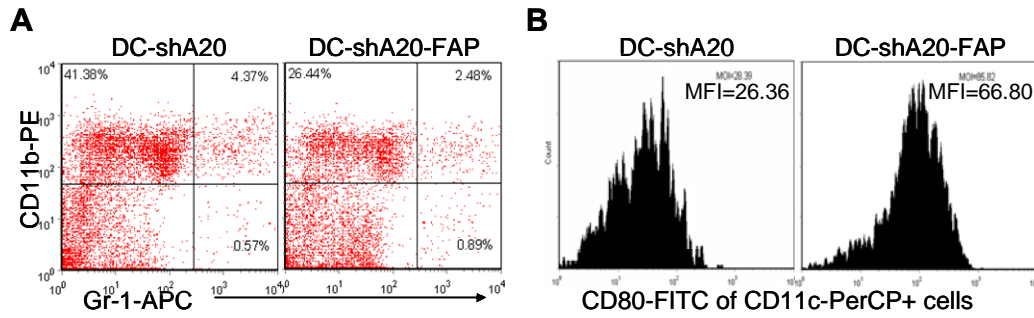


Fig.4. Influence of FAP-targeted DC vaccine on accumulation and maturation of macrophages and dendritic cells. C57BL/6 mice were inoculated s.c. with B16 cells. When the tumor sizes reached >5mm in diameter, mice were immunized with 1×10^6 DC-shA20-FAP, or DC-shA20. At 7 days after vaccination, tumor infiltrating cells were subjected to flow analysis. **A.** Dot plots show the effect of DC-shA20-FAP on the proportion of CD11b⁺Gr1^{dim} and CD11b⁺Gr1^{hi} cells. **B.** Histograms show CD80 expression in CD11c⁺ gated tumor infiltrating cells with the MFI of CD80 indicated.

DC-shA20-FAP vaccine enhances CD8 infiltration in the tumor. We also investigate whether DC-shA20-FAP vaccine enhance CD8 infiltration in B16 tumors. Staining for CD8 showed that there was a dramatic increase in CD8⁺ T cells within the tumor after DC-shA20-FAP vaccination. These changes were statistically significant and represented a 3-fold (7.72%:19.31%) change in CD8⁺ proportion in the tumor (**Fig. 10**). These data support our hypothesis in Aim.1.C that A20-silenced FAP-targeted DC vaccine has the potential to enhance the infiltration and proliferation of tumor-specific T cells at the tumor site.

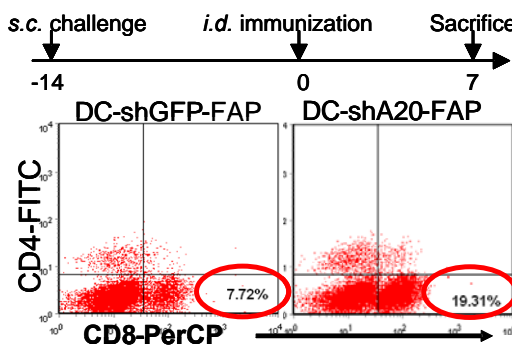


Fig.10. DC-shA20-FAP enhances CD8⁺ T cell infiltration in the tumor. C57BL/6 mice were inoculated s.c. with B16 cells. When the tumor sizes reached >5mm in diameter, mice were immunized with DCs. 7 days later, tumor infiltrating cells were subjected to flow analysis.

C. Summary. The data described above demonstrated that DC-shA20-FAP-HER2 vaccine induce robust T cell responses against FAP or HER2, resulting enhanced antitumor effect.

Key Research Accomplishments

- Construction of lentiviral vector expressing mouse A20 shRNA, FAP, and HER2.
- Silencing of A20 in DCs could enhance FAP- and HER2-specific T cell immune responses.
- Silencing of A20 in DCs could enhance anti-tumor effect in 4T1.2-neu bearing mice.

Reportable Outcomes

1. Presentation: An A20-silenced, stroma-targeted DC vaccine reverses the immunosuppressive tumor microenvironment resulting in potent antitumor activity. Zhu W, Zhou X, Rollins L, Rooney CM, Gottschalk S, Song XT. Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope Meeting, August 2-5, 2011. Orlando Marriott World Center Hotel, Orlando, FL.

Conclusions

- Demonstrates critical role for A20 in regulating the extent of T cell responses against FAP and HER2
- Providing an effective approach for developing potent DC vaccine against breast cancer.

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Appendices

Abstract:

An A20-silenced, stroma-targeted DC vaccine reverses the immunosuppressive tumor microenvironment resulting in potent antitumor activity. Zhu W, Zhou X, Rollins L, Rooney CM, Gottschalk S, Song XT. Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope Meeting, August 2-5, 2011. Orlando Marriott World Center Hotel, Orlando, FL.

Appendix

An A20-Silenced, Stroma-Targeted DC Vaccine Reverses the Immunosuppressive Tumor Microenvironment Resulting in Potent Antitumor Activity

Wei Zhu, Xiaou Zhou, Lisa Rollins, Cliona M. Rooney, Stephen Gottschalk, Xiao-Tong Song. Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children's Hospital, The Methodist Hospital, Houston, TX;

Dendritic cell (DC) vaccines have produced disappointing benefits in most clinical studies of cancer. This is most likely due to the presence of immunosuppressive cells within the tumor. Inhibition may come from regulatory T cells (Tregs) and also from the tumor supporting stroma, since cancer associated fibroblasts (CAFs; the central component of the tumor stroma) secrete inhibitory cytokines. Moreover, the extracellular matrix itself may protect malignant cells from effective immunity. We have previously shown that an A20-silenced DC vaccine renders DC resistant to Treg mediated immunosuppression and the aim of this project was to evaluate the effects of an A20-silenced DC vaccine targeting fibroblast activating protein (FAP) expressed on CAFs in the murine 4T1-neu breast cancer model. Bone marrow derived DCs were genetically modified to express an A20-specific shRNA, murine FAP, and rat HER2 (DC-shA20-FAP-HER2). DC-shA20-FAP-HER2 vaccination induced potent CD4⁺ and CD8⁺ FAP- and HER2-specific T-cell responses in BALB/c mice as judged by IFN- γ Elispot assays. DC-shA20-FAP-HER2 vaccination resulted in potent antitumor effects in prophylactic and therapeutic 4T1-neu models. Vaccinating tumor bearing mice with DC-shA20-FAP induced a significant decrease of tumor-infiltrating macrophages and myeloid-derived suppressor cells in comparison to DC-shA20 vaccinated mice. Conversely, DC-shA20-FAP vaccination induced a 2-fold upregulation of CD80 expression on tumor-infiltrating DCs as well as an increase of infiltrating CD8⁺ T cells. We show here that an A20-silenced, FAP-targeted DC vaccine induces potent FAP-specific immune response, reverses the immunosuppressive tumor microenvironment and has potent antitumor activity for breast cancer. Thus, targeting the tumor stroma has the potential to improve current DC vaccine approaches for breast cancer.

Keywords: Immunotherapy; Cancer vaccine; Animal Models